Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

An enzymatic biofuel cell utilizing a biomimetic cofactor

Elliot Campbell,[a] Matthew Meredith,[b,c] Shelley D. Minteer,[b,c] and Scott Banta*[a]

[a] Department of Chemical Engineering, Columbia University in the City of New York, New York, NY 10027, USA. Fax: 1 212 854 3054; Tel: 1 212 854 7531; E-mail: sbanta@columbia.edu. [b] Department of Chemistry, Saint Louis University, St. Louis, MO 63103, USA. [c] Present address: Department of Chemistry and Materials Science and Engineering, 315 S 1400 E Room 2020, Salt Lake City, UT 84112, USA. E-mail: minteer@chem.utah.edu

Experimental Section

Chemicals: NAD^+, NMN^+, neutral red, Nafion® 1100EW suspension, 2,3-butanediol (mixture of DL and meso) and all salts were purchased from Aldrich and used as received. Nafion® modified with tetrabutylammonium bromide (modified Nafion®) was prepared according to a previous protocol.[1] All other chemicals were from Sigma-Aldrich and used without modification.

Protein Expression and Purification: Expression and purification of wt AdhD, K249G AdhD, H255R AdhD, and the K249G/H255R AdhD double mutant followed a previously established protocol.[2] Purity was assessed by SDS-PAGE and standardized activity assays, and purified enzyme was stored lyophilized. All enzyme concentrations were determined from A_{280} measurements with a calculated molar extinction coefficient of ε_{280} = 52495 M^{-1} cm^{-1} and confirmed using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

Fluorescence Titrations: Dissociation constants for the enzyme-cofactor complexes were determined by fluorescence titration.[3-5] Briefly, 2 µM enzyme in 50mM glycine (pH 8.8) was stirred in a 1 cm quartz cuvette placed in a J-815 spectrometer (Jasco Inc., Easton, MD) equipped with a Peltier junction temperature control. Samples were excited at 280 nm, and the fluorescence quenching upon cofactor binding was monitored at 330 nm. The total volume of cofactor added was less than 1% of the reaction volume to limit dilution effects. Experiments were repeated in at least triplicate, and data were fit to a saturation adsorption isotherm.

Steady-state Kinetics: The full kinetic parameters for the K249G/H255R double mutant AdhD were determined with both NAD^+ and NMN^+ using 2,3-butanediol or D-arabinose as a substrate. Initial rates at 25°C were measured using a SpectraMax M2 plate reader by following the production of the reduced cofactor at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}). Reactions contained 50 mM glycine (pH 8.8), 1-100 mM substrate, and the appropriate amount of enzyme and were initiated with 1-1000 µM NAD^+ or 50-2500 µM NMN^+. Data were collected in at least triplicate, and were fit simultaneously to the ordered bi-bi rate equation[6] (Eq. 1) using non-linear least-squares regression. This reaction mechanism was previously verified.[7] Reported errors are standard deviations.

$$V = \frac{E_t k_{cat} AB}{K_{ia} K_B + K_B A + K_A B + AB} \qquad (1)$$

Electrode Fabrication: Glassy carbon rotating disc electrodes (5 mm diameter) were purchased from Pine Instruments and a Pine Rotator (Model AFM-SRX) was used for all rotating experiments. Electrochemical measurements were taken with a CH Instruments model 810 potentiostat interfaced with a PC. All voltammetric experiments were carried out using a platinum mesh counter electrode and a saturated calomel reference electrode (SCE). Neutral red was electropolymerized according to previously published protocols[8, 9] as follows: Neutral red (11.5 mg) was dissolved into 100 mL of a pH 6 buffer solution consisting of 0.25 M phosphate and 0.1 M $NaNO_3$. The potential was swept at a scan rate of 50 mV/s between 0.8 V and -0.8 V (vs. SCE) for 6 complete cycles (12 scans). After electropolymerization, the electrodes were carefully washed with 18MΩ water to remove any residual monomer and dried under a gentle stream of nitrogen. Modified Nafion® (10 µL of a 5% by wt suspension in 100% ethanol) was drop-cast on top of the poly(neutral red) (PNR)-modified GC electrodes and allowed to dry for 4-6 hours.

These modified electrodes were then soaked in Tris buffer solutions (10 mM Tris-HCl, 10 mM KCl, pH 7) containing 10 mM of either NAD^+ or NMN^+ for 18 hours before use.

Biofuel cell anodes utilizing poly(methylene green) (PMG) as an electrocatalyst were prepared similarly to previously published procedures[10] as follows: Methylene green was polymerized onto 1 cm^2 pieces of Toray paper (TGP-060, E-Tek) by performing cyclic voltammetry (6 complete cycles, -0.3 V to 1.3 V) at a scan rate of 50 mV/s in a degassed solution containing 0.4 mM methylene green, 0.1 M sodium nitrate, and 10 mM sodium borate. The electrode was rinsed and then allowed to dry overnight. Enzyme/Nafion® casting solutions (50 µL of 5 wt% modified Nafion® in 100% ethanol combined with 150 µL of pH 7.4 phosphate buffer containing 1 mg/mL enzyme and 1 mg/mL NAD^+ or 0.5 mg/mL NMN^+) were pipetted in 50 µL aliquots onto each PMG-modified electrode and allowed to dry for 4-6 hours. The bioanodes were soaked in a fuel solution (pH 8.0) consisting of 50 mM phosphate, 100 mM sodium nitrate, 50 mM arabinose, and 1 mM NAD^+ or NMN^+ overnight before use in the biofuel cell.

Biofuel Cell Testing: Bioanodes were tested in a biofuel cell apparatus that has been previously described.[10] The cell consisted of two vertical glass chambers, separated by the cathode, which was coated with a Nafion® polymer electrolyte membrane. The upper glass chamber contained the fuel solution, and the bottom chamber was open to the air to allow O_2 to flow to the cathode. The cathode material was an ELAT electrode with 20% Pt on Vulcan XC-72 (E-Tek). The cathode was hot pressed to the backside of a Nafion® NRE 212 PEM with the catalyst side facing the membrane for 1 minute. The fuel solution was identical to the anode soaking solution, consisting of 50 mM sodium phosphate, 100 mM sodium nitrate, 50 mM arabinose, and 1 mM NAD^+ or NMN^+. Data was collected using a CH Instruments model 810 potentiostat. The reference and counter electrodes were connected to the bioanode, and the working electrode was connected to the cathode. The biofuel cell was allowed to reach a steady open circuit potential, after which a polarization curve was taken at a scan rate of 1 mV/s.

Cyclic Voltammetry and Rotating Disc Voltammetry: Cyclic voltammetry was carried out at various scan rates on each electrode in the pH 7 Tris buffer, using a potential window of 0.2 V to -0.8 V. Rotating disc voltammetry was carried out at various rotation rates on each electrode at a scan rate of 10 mV/s. Electrodes were rotated for 10 minutes at each rotation rate before the voltammetry was performed.

Table S1 – Apparent Kinetic Parameters of wt AdhD and Mutants

	Enzyme	k_{cat} (min^{-1})	K_M (μM)	K_D (μM)	k_{cat}/K_M (x 10^{-5} μM^{-1} min^{-1})
NMN^+	wt AdhD	0.03	2500	875	1.2
	H255R	0.07	2100	ND	3.3
	K249G	0.8	3800	ND	21
	K249G/H255R	1.6	2600	826	62
NAD^+	wt AdhD	85	60	59	140000
	K249G/H255R	180	460	45	40000

Reaction conditions: 50mM glycine, 100mM 2,3-butanediol (pH 8.8), 45°C. Reactions were initiated by the addition of NAD^+ (1 - 1000 μM) or NMN^+ (5 – 3000 μM), and monitored at 340nm. Each experiment was run in triplicate, and the data was fit to a simplified rate equation (Eq. S1). K_Ds were obtained by fluorescence titration, as previously described.

$$v = \frac{k_{cat}^{app} E_t A}{K_M^{app} A} \quad \text{Eq. S1}$$

Table S2 – Steady-State Kinetic Parameters

	Cofactor	k_{cat} (s^{-1})	K_{ia} (μM)	K_A (μM)	K_B (mM)
wt AdhD					
2,3-butanediol	NAD^+	1.0 ± 0.1	37 ±2	63 ±2	29 ±1
	NMN^+	<0.0005	ND	ND	ND
K249G/H255R AdhD					
2,3-butanediol	NAD^+	15 ± 2	11 ± 1	460 ± 60	690 ± 80
	NMN^+	0.018 ± 0.002	880 ± 10	140 ± 20	17 ± 5
D-arabinose	NAD^+	65 ± 1	78 ± 3	480 ± 10	72 ± 2
	NMN^+	0.55 ± 0.03	1700 ± 100	1100 ± 100	130 ± 10

Table S3 – Properties of Nicotinamide Cofactors in Nafion® Films

Cofactor	Diffusion Coefficient (cm^2/s)	Extraction coefficient
NAD^+	5.45 (± 0.37) x 10^{-9}	1.15 ± 0.09
NMN^+	4.32 (± 0.43) x 10^{-8}	0.44 ± 0.05

Table S4 – Summary of Biofuel Cell Performance

Cofactor	Open Circuit Potential (V)	Max Power Density (μWatt/cm^2)	Max Current Density (μA/cm^2)
NAD^+	0.642 ± 0.011	1.52 ± 0.27	16.1 ± 3.6
NMN^+	0.593 ± 0.069	1.37 ± 0.24	22.8 ± 2.6

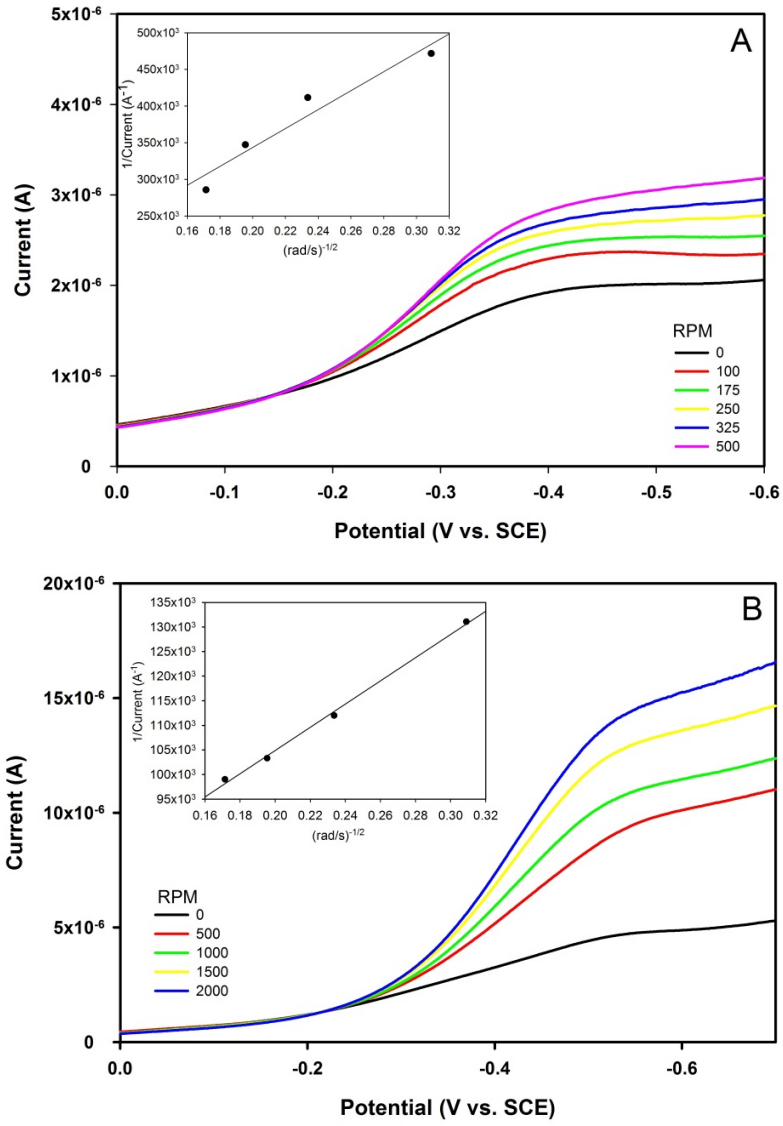

Figure S1. Representative rotating disc voltammograms of NAD^+ (A) and NMN^+ (B) at a PNR-modified GC electrode coated with modified Nafion® at a variety of rotation rates. Conditions: Quiescent solution, room temperature, 10 mM Tris-HCl, 10 mM KCl, 10 mM NAD^+/NMN^+, pH 7. Insets: Koutechy-Levich plots showing a linear relationship between inverse current and inverse square root of rotation rate.

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